(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 May 2001 (17.05.2001)

PCT

(10) International Publication Number
WO 01/34832 A2

(51) International Patent Classification[7]: C12Q 1/32, 1/26

(21) International Application Number: PCT/GB00/04283

(22) International Filing Date:
8 November 2000 (08.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9926435.0 8 November 1999 (08.11.1999) GB

(71) Applicant *(for all designated States except US)*: **POLYMER LABORATORIES LIMITED** [GB/GB]; 115 Colmore Row, Birmingham B3 3AL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HOBBS, Roderick, Nicholas** [GB/GB]; Polymer Laboratories Limited, Essex Road, Church Stretton, Shropshire SY6 6AX (GB). **MARGETTS, Graham, Ian** [GB/GB]; Polymer Laboratories Limited, Essex Road, Church Stretton, Shropshire SY6 6AX (GB).

(74) Agents: CHAPMAN, Paul, W. et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/34832 A2

(54) Title: METHOD

(57) Abstract: Novel analytical/diagnostic methods are provided. Diagnostic reagent material for use in the novel methods is also described. The methods/reagents find particular use in redox enzyme catalysed reactions.

METHOD

The present invention relates to improved analytical and/or diagnostic methods, as well as materials for use in such methods. In particular, the methods and materials find use in clinical diagnostic assays based on measuring redox reactions, e.g. measuring analytes such as transaminases, glucose and lactate.

Many current diagnostics of clinical and other biological application exploit redox reactions for measuring analytes in biological fluids, for example blood glucose and serum transaminase. The methods upon which these diagnostics are based use one or more redox enzymes as catalysts of reactions which modify the chemical state of indicator compounds producing measurable changes in these indicators.

Tetrazolium dyes are widely used as indicators for diagnostics because they are essentially colourless and change into brightly coloured formazans upon reduction by redox catalysts. Although tetrazolium salts and their reduction thereof have been used for over one century in industrial chemistry, histochemistry and biochemistry (for a review, see Seidler E (1991) Progress Histochem. Cytochem. 24, 1-86), their application in clinical diagnostics is continuously expanding.

For instance, tetrazolium applications have been commercially developed for dry reagent diagnostics such as glucose (US patent No. 5,278,047), and recently also for immunoassays (US patent No. 5,916,746). Despite their popularity and usefulness, tetrazolium indicators suffer from various problems limiting their applications in commercial diagnostics.

A major problem is that tetrazolium compounds react non-specifically with many substances present in biological fluids and assay systems (Stuart et al. (1975) Histochem. J. 7, 471-487; Seidler (1991) Progress Histochem. Cytochem. 24, 1-86).

The consequent interference in optical measurements is very troublesome in clinical and biological assays and several patents have been granted which try to address these problems and expand the practical applications of tetrazolium indicators (US patents Nos. 5,013,647 ; 5,166,049 ; and 5,902,731). None of the reported procedures for limiting the non-specific reactions of tetrazolium salts is fully satisfactory, especially in dry reagent diagnostics which require high concentrations of the indicators for rapid colour development (US patent No. 5,250,695).

Another problem that further limits the application of tetrazolium indicators to whole blood diagnostics is their interaction with blood cells, since both white and red cells take up the indicators and reduce them via intracellular redox systems (Stuart et al. (1975) Histochem. J. 7, 471-487). In order to limit these non-specific reactions with blood cells, diagnostics based on strips and dry reagents often contain complex and expensive structures with layers of porous material that can separate cells from the blood fluids (US patents No. 5,360,595 and No. 5,902,731).

An inconvenience well known to those skilled in the biochemical art is that most of the brightly coloured formazans produced by the reduction of tetrazolium salts precipitate out of the assay solution (Seidler (1991) Progress Histochem. Cytochem. 24, 1-86; Haugland (1996) Handbook of Fluorescent Probes, Sixth edition, pp. 494-497, Molecular Probes Inc., Eugene, OR). The water solubility and stability of tetrazolium salts can be improved by chemical modifications that are of special utility for dry diagnostics technology (US patents Nos. 5,250,695, and 5,583,006). Unfortunately, tetrazolium dyes with water-soluble formazans such as 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) and derivatives are not commercially available, or are comparatively expensive, and generally produce red-purple colours that are inconvenient for diagnostic assays using whole blood samples.

The majority of analytical applications preferentially require commercially available and cheap tetrazolium salts such as Nitro Blue Tetrazolium (NBT) which, owing to the poor

water solubility of their coloured products, stick to plastic or other supports and thus complicate the design and method of current and novel assays.

Although the precipitation of coloured formazans can be reduced by the addition of surfactants, especially in dry diagnostic formulations, it remains a major concern in the development of new assays of biological analytes because it prevents an easy separation of the coloured indicator from the reaction mixture and its support/container.

Redox enzymes collectively called diaphorases (NADH or NADPH: acceptor oxido-reductases) are the preferred catalysts for reducing tetrazolium indicators in diagnostic assays. Bacterial lipoamide dehydrogenases are most commonly used as diaphorases since they oxidise NADH and NADPH with similar efficiency. The usefulness of lipoamide dehydrogenases and other diaphorases in diagnostics is hampered by the limited stability of their activity in aqueous solution, which is normally compensated by the costly expedient of using high enzyme concentrations in the formulation of dry reagent systems (US patent No. 5,902,731).

Other problems limiting the usefulness of diaphorase enzymes in diagnostics arise from the fact that their redox reaction with tetrazolium salts in solution is non-physiological and in part mediated by oxygen radicals (Seidler (1991) Progress Histochem. Cytochem. 24, 1-86). Immobilisation of bacterial diaphorases to synthetic supports usually improves the stability of their redox activity with indicators such as dichloroindophenol (DCIP) (e.g. Lowe (1977) Eur. J. Biochem. 76, 401-409).

There is therefore a continuing need to provide improved materials and methods which will allow the use of the convenient tetrazolium indicators as well as diaphorase enzymes. We have now developed such materials and methods which allow assays to carried out quickly, with low enzyme concentrations and which allow detection of very low analyte concentrations. In addition, the methods of the present invention can also

carried out using whole blood samples, without the necessity of removing the blood cells themselves.

Thus, in a first aspect, the present invention provides a diagnostic reagent material comprising one or more polymers, combined with one or more redox catalysts, wherein the one or more polymers are hydrophilic polymers with free functional groups, e.g. free amine groups.

Surprisingly, we have found that the use of such a diagnostic reagent material, when using tetrazolium indicators, overcomes many of the disadvantages described above. In particular, coloured reaction product is adsorbed or fixed by the polymer material, concentrating the coloured product. The reaction is rapid and extremely sensitive. In fact, the methods described herein achieve local amplification and dye accumulation within the material.

As defined herein the polymer is hydrophilic in character with free functional groups, e.g. free amine groups. In the context of the present invention, the abolute degree of hydrophilicity of the one or more polymers is less important than that the polymer(s) should possess the property of swelling in aqueous media. An example of such a polymer is Polyethylene Glycol Acrylamide (PEGA).

The one or more redox catalysts can be any form of redox catalyst, including chemical agents such as phenazine methosulphate (PMS) or enzymes such as diaphorases. Examples of suitable diaphorases include lipoamide dehydrogenase, alcohol dehydrogenase, NAD(P)H-quinone oxidoreductase and ferredoxin-NADP reductase. Lipoamide dehydrogenase from micro-organisms, including thermophilic bacteria, represents a preferred embodiment of the invention.

In the context of the present invention, the term "combined with" includes chemical conjugation, e.g. using well known condensing or cross-linking reagents, such as

glutaraldehyde or co-polymerisation of a suitably modified redox catalyst, non-covalent conjugation, e.g. by hydrogen bonding and physical entrapment within a polymer matrix. Entrapment can be achieved by polymerisation of precursor component of the polymer in the presence of the redox catalyst such that the catalyst is physically entrapped within the polymer network. Entrapment may be enhanced by making polymeric forms of redox enzymes.

However, in practice, the skilled person will appreciate that combination of the catalyst with the material could be achieved by a mixture of the above. Chemical and/or hydrogen conjugation is the preferred embodiment of the claim.

In a preferred embodiment the polymeric material is in the form of beads or particles. Such beads or particles will find use in many "conventional" diagnostic/analytical applications. They could, for instance simply be mixed with a sample to be tested, to which in turn suitable reagents are added. In addition, they would also find use in so-called "lateral flow" diagnostics, where the beads would be provided in a dry state and the addition of a test sample entrains the beads or particles and carries them to another area of a test strip. Suitably, when the material is in the form of beads, the size of the beads will range from 0.0001 to 2mm, preferably from about 0.1 to 0.5mm.

The enzyme polymer matrix may be further entrapped with in a column, porous bag or membrane allowing ease of diffusion of analytes and substrates from a large volume of sample to the beads, whilst constraining the signal generating beads.

The material of the invention will also find use in the form of a membrane, mesh, film, gel or any other form suitable for dry reagent formulations including forms useful in sticker diagnostics for the testing of external body fluids such as sweat.

In a further embodiment, the polymer is conjugated to at least two enzymes, forming a dual enzyme particle, allowing for fast and efficient colour production in such assay

systems. An example of such a system is that consisting of lactate dehydrogenase and a diaphorase for use in the assay of lactate in biological fluids.

As discussed above, diaphorases are relatively unstable in aqueous solution. We have found that the material of the present invention improves stability of diaphorase enzymes, such that much lower concentrations can be used in the assay reactions. Thus, in a second aspect, the invention provides a diagnostic reagent material comprising one or more polymers, conjugated to one or more diaphorase enzymes, wherein the one or more polymers are hydrophilic polymers with free functional groups, e.g. free amine groups. Preferably, the material is in the form of beads or particles. In a particularly preferred embodiment of this aspect of the invention, the immobilised enzyme is lipoamide dehydrogenase and the polymeric material is beads of polymerised polyethylene glycol acrylamide, which achieves a remarkable stability of the diaphorase activity.

In a third aspect the present invention provides the use of the diagnostic reagent material in a method for the detection or quantitation of an analyte.

As discussed herein the material of the present invention has certain advantages when used in diagnostic and/or detection methods involving redox catalysts. Thus, in a fourth aspect the present invention provides a method for the detection and/or quantitation of an analyte which comprises the step of bringing a sample to be tested into contact with diagnostic reagent material of the invention, in the presence of one or more reagents required for the reaction. In the context of the present invention, the term reagents relates to one or more of the following: indicators such as chromogenic or fluorogenic dyes, including tetrazolium salts such as Nitro Blue tetrazolium (NBT); substrates; redox substrates, such as NADH or NADPH; buffers; stabilisers, such as EDTA; and/or additional catalysts, eg redox catalysts such as diaphorases or other enzymic, or non-enzymic catalysts.

The sample to be tested can be any suitable biological or non-biological sample. In the case of the former, suitable types of sample include urine, whole blood, sweat, saliva etc. In addition, it will also be possible to use solid or semi-solid samples with the material of the invention. The skilled person will appreciate that the form of the material to be used will depend, to some extent, on the type of sample being analysed/tested. As discussed herein the methods of the present invention are particularly useful in testing whole blood, since the sample does not require pre-treatment to remove blood cells prior to testing. However, other samples may require pre-treatment and such pre-treatment can be included in the methods of the invention.

As discussed herein, the methods of the present invention can suitably be used to detect/quantitate analytes based on redox reactions. Examples of such analytes include transaminases and glucose.

Suitably, the diagnostic reagent material of the invention can be provided as part of a kit for use in carrying out diagnostic, detection or quantitation assays. Thus, in a further aspect the present invention provides a kit comprising the diagnostic reagent material of the invention together with one or more reagents suitable for carrying out one or more diagnostic, detection or quantitation assays.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*. The invention will now be described with reference to the following examples, which should not in any way be construed as limiting the scope of the invention.

Example 1

PEGA beads with size ranging from 0.1 to 0.5 mm are obtained essentially according to methods disclosed in US patent No. 5,352,756, which is hereby incorporated by reference, and preserved in methanol or water mixture thereof. After washing out the

methanol by centrifugation in aqueous media, preferably containing buffers with pH close to neutrality such as phosphate-buffered saline (PBS), the PEGA beads are suspended in a few ml of the same buffered medium and treated with a fresh solution of glutaraldehyde in PBS for some hours under constant stirring. The final concentration of glutaraldehyde in the solution ranges from 0.2 to 2 %, and preferably from about 0.3% to 1%; the time of treatment varies from about 1 to 4 hours, and preferably is of about 1 to 3 hours.

Excess glutaraldehyde is subsequently removed by several washes in a buffered medium such as PBS, and the washed beads are mixed with bacterial lipoamide dehydrogenase freshly dissolved in PBS, and incubated overnight at around 4 °C.
The final concentration of diaphorase in the incubation with PEGA beads ranges from 0.05 to 1 mg/ml, and preferably is between 0.1 and 0.3 mg/ml. Other redox enzymes such as glutamate dehydrogenase or lactate dehydrogenase can also be added to obtain successful conjugation to PEGA beads together with diaphorase. For instance, a dual enzyme particle reagent having a ratio ranging from 10 to 100 between the activity of lactate dehydrogenase and diaphorase is very effective in applications for the assay of lactate. The incubation is stopped by dilution with a buffered solution containing from 0.1 to 1 M Tris-Cl and with pH varying from 7 to 9, for instance 0.2 M Tris-Cl pH 8, and unbound diaphorase is removed by centrifugation with several washes of Tris-Cl buffered media with pH around 8.

The final pellet of PEGA beads with conjugated diaphorase is resuspended in Tris buffer, with molarity preferably ranging from 0.01 to 0.1 M and pH between 7 and 9, and stored at temperatures around 4 °C. Figure 1 shows that the specific activity of diaphorase increases in the first few days after conjugation to PEGA beads according to the method in above, and then decreases slightly in subsequent weeks. On the contrary, a parallel sample of diaphorase diluted to the same final concentration in Tris medium loses its activity completely within a few days. Hence, the stability of diaphorase

activity is strongly increased after conjugation to PEGA beads, and can be extended to months when refrigerated.

As part of this invention, a method has been developed to assay the diaphorase activity following known art in the biochemical field and used for the activity assays in the experiment shown in Fig. 1.

In this method, a solution with the following composition is prepared.

Tris-Cl, pH about 8	50	mmol/l
EDTA	5	mmol/l
DCIP	0.05	mmol/l
NADPH	0.2	mmol/l

0.9 ml of this solution is transferred in test tubes containing 0.1 ml of Tris-Cl buffer (blank) or 0.1 ml of a solution of either PEGA beads with conjugated diaphorase, or diaphorase diluted in Tris buffer (to a final concentration ranging between 0.0001 and 0.01 mg diaphorase protein per ml of assay solution). After mixing, the solution is left at room temperature (20 to 30 °C) for 5 min and then 0.2 triplicate aliquots are transferred to a photometric plate reader and the Absorbance at 570 nm is measured.

The activity is calculated according to the formula:

Diaphorase activity (units)= $\Delta A \times 0.2 / 8.2 \times [enz]$

Where ΔA represents the absorbance change in 5 min minus the corresponding change in the blank sample, 8.2 corresponds to the millimolar extinction coefficient of DCIP at 570 nm normalised to the pathlength of the plate reader well, and [enz] is the diaphorase concentration in mg per ml. Variations of the above method for the diaphorase assay include the use of different concentrations (from 0.01 to 1 mM) of either NADPH or NADH, changes in Tris

molarity from 0.01 to 0.5 M with pH values ranging from 6 to 10, EDTA concentrations from 0.01 to 10 mM, and measurements of DCIP reduction at 600 nm in standard spectrophotometers, in which case a millimolar extinction coefficient of 21/1cm pathlength is routinely used (Lowe (1977) Eur.J.Biochem. 74, 401-409).

This invention provides another method for measuring diaphorase activity derived from the above method, but using various tetrazolium salt indicators. This method is particularly suited for diagnostic assays in which NADH or NADPH is produced from their corresponding oxidised cofactor by a combination of enzymes added to the assay mixture for obtaining the indirect measurement of analytes present in the test fluid, for example transaminases. The following method yields a rapid and highly specific production of formazan indicators within the particle reagent; the formazans accumulated in the particles can be separated from the assay mixture and either extracted for photometric quantitations or directly evaluated by colour reflectance, for instance after transferring onto white supports.

The method requires a buffered solution containing any buffer system with pH ranging from 6 to 9, and molarity ranging from 0.02 to 0.2 M, for instance Tris-Cl 0.05 M, pH about 8.0. This solution may also contain EDTA in concentrations ranging preferably from 2 to 6 mM. A fresh solution of either NADPH or NADH is added to a final concentration ranging between 0.001 and 1 mM, and preferably between 0.1 and 0.4 mM. A particle reagent containing conjugated diaphorase is added to a volume ranging between 0.04 and 0.2 (v/v) of the final reaction volume of the buffered solution. Finally, an aqueous solution of a tetrazolium salt is added to a final concentration of about 0.05 to 1 mM. Although the order of reagent addition is of no particular importance, the reaction is normally started by tetrazolium addition and allowed to proceed at room temperature (20 to 30 °C) for times ranging from 2 to 40 min.

The particle reagent is then separated by centrifugation; the supernatant is removed and may be used for optical measurements. The coloured pellet is either extracted with

solvents such as DMSO, or solutions containing high salts, or transferred to appropriate supports for reflectance measurements.

Tetrazolium salts include, but are not restricted to, 4-Iodonitrotetrazolium violet (INT), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), XTT, NBT and Tetranitro blue tetrazolium (TNBT). The wavelength for the optical measurement of the supernatant is usually set at 490 nm (for INT and XTT) or 570 nm (for MTT, NBT and TNBT).

Using particle reagents of large size, e.g. 0.1 to 1 mm, the isolation of the formazan colour accumulated within the particle is easily achieved by separation of the particle reagents from the other components of the test fluid that could interfere with the measurement. These large particle reagents offer the additional advantage of preventing interference from intracellular reactions of tetrazolium dyes, since they are too big to be actively taken up by blood cells or other particulate biological material. The accumulation of formazan indicators in the particle reagent is normally reversible, but can be also irreversible depending on the composition of the particle reagent, the type of tetrazolium indicator, and the assay method used.

Using particle reagents formed by PEGA-conjugated diaphorase as in Example 1 above, the formazans of tetrazolium salts such as MTT or INT bind reversibly through predominantly hydrophobic interactions. Hence, they can be extracted with DMSO or other organic solvents. Using particle reagents formed by diverse polymers with conjugated diaphorase, the water-soluble formazans of tetrazolium salts such as XTT bind to the particles also through hydrophilic interactions, and can be efficiently extracted not only with DMSO, but also with high osmolarity media, e.g. 1M Tris-Cl. Surprisingly, the formazans of tetrazolium salts such as NBT and TNBT bind to particle reagents formed by PEGA-conjugated diaphorase in a way which produces reversibly bound intermediates and also irreversibly bound coloured products. This is especially the case using the example method described below.

Example 2

The method involves the preparation of samples in any suitable labware container, e.g. Eppendorf tubes, with the following reagents.

Tris-Cl	50 mM, pH 8	0.41 ml
EDTA	250 mM	0.01 ml
NADPH	10 mM	0.02 ml
NBT	10 mM	0.01 ml
PEGA-diaphorase reagent		0.05 ml

The PEGA-diaphorase reagent is obtained as explained in Example 1 and may contain from about 0.001 to 0.02 mg/ml of diaphorase protein.

The reagents are mixed and allowed to react for 10 min at room temperature, and the particles are then separated by centrifugation, for instance at 2000 to 5000 RPM for times of about 2 to 10 min with a bench centrifuge. Parallel samples are prepared in the same way except that the PEGA-diaphorase particle reagent is substituted with either 0.05 ml of Tris-Cl buffer (blank) or 0.05 ml of a 50% solution of PEGA beads without diaphorase conjugation. Whereas the PEGA beads with conjugated diaphorase become brightly blue within about 2 min of reaction, those without diaphorase remain colourless for hours, and become pale blue only after 12 or more hours of reaction. At these time also the blank produces some insoluble blue formazan due to the slow non-specific reaction between NADPH and NBT.

During the reduction catalysed by diaphorase bound to the PEGA beads following this method, some tetrazolium compounds such as NBT form stable intermediates of purple colour which bind in a reversible way, since they are extracted with DMSO. The purple colour extracted by DMSO is proportional to the quantity of NADPH in the assay mixture, and may thus be used for quantitation. Unexpectedly, when tetrazolium compounds contain nitrophenyl substituents such as in NBT, the final reaction products bind with a tight, irreversible binding of the bright blue formazans to the PEGA particle reagent. As a result of this unexpected reaction, the particle reagent become permanently coloured in bright blue, since the bound

formazans cannot be removed by repeated extractions with DMSO or other procedures (including detergents).

Especially with PEGA-conjugated diaphorase reagents obtained as described in Example 1, the diaphorase reaction produces a highly specific colour that is locally amplified by accumulation of dyes such as NBT formazan within the same particle reagent. This has clear advantages over the formazan production in solution that is normally measured in current diagnostic assays. In particular, interferences due to non-specific reactions of tetrazolium indicators with other components of the test fluids are minimised by the high local concentration of the tetrazolium substrate and the diaphorase enzyme which occurs within the porous matrix of particles formed by polymers such as PEGA. Owing to the limited size of the matrix pores, the diaphorase reaction is also shielded from macromolecules or cells present in blood or other particulate biological fluids that can be used in diagnostic tests.

The accumulation, amplification and fixation of the colour indicator within the particles overcome many inconveniences due to the poor water solubility of formazans such as those of NBT and TNBT, and hence render superfluous the inclusion of surfactant compounds in the reaction mixture. Moreover, the size of the particle reagents enables their rapid separation from the assay mixture by techniques such as centrifugation, lateral flow, filtration, magnetic separation, and immuno-capture, thereby enabling a facile quantitative or qualitative evaluation of diagnostic reactions producing NADH or NADPH.

Three detailed examples are now presented for applications of the particle reagent obtained as detailed in Example 1 and the method described in Example 2 above to the assay of analytes of clinical and diagnostic importance. The first application describes the determination of a clinically relevant blood transaminase, aspartate amino-transferase (AST or GOT).

Example 3

To a final volume of 0.6 ml, the following reagents are added at the final concentration indicated, and with an overall pH around 8.

Tris-Cl	50 mM
Na-EDTA	5 mM
Na-aspartate	40 mM
Na-oxoglutarate	0.8 mM
NADP	1 mM
Glutamate dehydrogenase	10 units/ml
NBT	0.15 mM
PEGA-diaphorase reagent	1:10 dilution

The reagents are mixed and equilibrated at room temperature and then the reaction is started with a small volume, usually ranging between 0.01 and 0.05 ml, of either a solution of pure aspartate aminotransferase (AST) or a biological fluid containing variable amounts of the said AST.

The concentration of aspartate amino-transferase may vary between 10 and 1000 units per litre of assay mixture and the time of reaction may vary between 2 and 30 min at room temperature (20 to 30 °C). Longer times are preferred for the lowest concentrations of the test AST and shorter times for the highest concentrations of the said enzyme. The blue coloured particles obtained after 20 min reaction with the protocol of Example 3 are shown in the photograph of Figure 2. In the specific experiment shown in Figure 2, the concentration of AST has been varied between 25 and 500 U/l, a range which encompasses the values usually found in normal and pathological human sera.

Another example application of the particle reagent of PEGA beads and NBT colour fixation relates to the determination of glucose in a biological fluid, for instance whole blood.

Example 4

To a final volume of 0.5 ml, the following reagents are added at the final concentration indicated, and with an overall pH around 8.

Tris-Cl	50 mM

Na-EDTA	5 mM
ATP	2 mM
Glucose kinase	10 units/ml
NADP	1 mM
Glucose 6-phosphate dehydrogenase	2 units/ml
NBT	0.2 mM
PEGA-diaphorase reagent	1:10 dilution

The reagent are mixed and equilibrated at room temperature and then the reaction is started with a small volume, usually ranging between 0.01 and 0.05 ml, of either a solution containing known amounts of D-glucose or a biological fluid containing variable amounts of D-glucose. The final concentration of D-glucose may vary between 1 and 200 mM in the assay mixture and the time of reaction varies between 2 and 10 min at room temperature (20 to 30 °C).

Variations in the method of Example 4 above allow a direct measurement of glucose-6-phosphate (from 0.1 to 5 mM in the assay solution). For this purpose, glucose kinase and ATP are omitted from the reaction mixture of the assay.

A further application of the present invention relates to diagnostics for sport and clinical medicine that evaluate the concentration of the lactate metabolite in body fluids, lactate accumulation deriving from either physical exercise, fatigue or metabolic malfunction, as for example in congenital myopathies. The example application refers to the determination of lactate in any of the following biological fluids from humans or animals (including horse and dog): whole blood, serum, urine, milk, saliva, sweat, tissue exudate. Moreover, the same method can be applied to the determination of lactate dehydrogenase in liquid media of cell cultures (either mammalian or microbial).

Example 5

To a final volume of 0.5 ml, the following reagents are added at the final concentration indicated, and with an overall pH around 8.

Tris-Cl	50 mM

Na-EDTA	5 mM
Lactate dehydrogenase	5 units/ml
NAD	1 mM
NBT	0.2 mM
PEGA-diaphorase reagent	1:10 dilution

The reagent are mixed and equilibrated at room temperature and then the reaction is started with a small volume, usually ranging between 0.01 and 0.05 ml, of either a solution containing sodium L-lactate, or a biological fluid containing variable amount of the L-lactate metabolite. The concentration of L-lactate may vary between 0.1 and 100 mM in the assay mixture, and the time of reaction varies between 2 and 10 min at room temperature (20 to 30 °C).
A dual enzyme particle reagent may replace both the soluble lactate dehydrogenase reagent and the PEGA-diaphorase reagent. When the dual enzyme particle reagent is prepared as described in Example 1, the production of NBT formazan in the beads is obtained with superior efficiency and speed, and thus is particularly suitable for measuring lactate in biological fluids such as saliva and sweat.

Variations of the method described in Example 5 can be applied to the assay of the enzyme lactate dehydrogenase in biological fluids such as serum, or in the culture media of human and animal cells. In these cases, lactate is present in the reaction mixture at concentrations of about 0.1 to 50 mM and specifically oxidised by the lactate dehydrogenase present in the test fluid with formation of NADH, which in turn is oxidised by diaphorase and colour formation with NBT.

CLAIMS:

1. A diagnostic reagent material comprising one or more polymers, combined with one or more redox catalysts, wherein the one or more polymers are hydrophilic polymers with free functional groups.

2. A material as claimed in claim 1 wherein the free functional groups are free amine groups.

3. A material as claimed in claim 2 wherein one of the one or more polymers is Polyethylene Glycol Acrylamide (PEGA).

4. A material as claimed in any one of claims 1 to 3 wherein the one or more redox catalysts are chemical agents or enzymes such as diaphorases.

5. A material as claimed in claim 4 wherein the chemical agent is phenazine methosulphate.

6. A material as claimed in claim 4 wherein the enzyme is a diaphorase.

7. A material as claimed in claim 6 wherein the diaphorase is lipoamide dehydrogenase, alcohol dehydrogenase, NAD(P)H-quinone oxidoreductase or ferredoxin-NADP reductase.

8. A material as claimed in claim 7 wherein the diaphorase is Lipoamide dehydrogenase derived from a micro-organism, eg thermophilic bacteria.

9. A material as claimed in any one of claims 1 to 8 wherein the material is in the form of beads or particles, a membrane, mesh, film or gel.

10. A material as claimed in claim9 wherein the beads or particles are further entrapped within a column, porous bag or membrane.

11. A material as claimed in any one of claims 1 to 10 wherein at least two redox enzymes are conjugated to the one or more polymers.

12. A material as claimed in claim 11 wherein the enzymes are lactate dehydrogenase and a diaphorase.

13. A diagnostic reagent material comprising one or more polymers, conjugated to one or more diaphorase enzymes, wherein the one or more polymers are hydrophilic polymers with free functional groups.

14. A material as claimed in claim 13 wherein the free functional groups are free amine groups.

15. A material as claimed in claim 13 or claim 14 wherein the material is in the form of beads or particles

16. A material as claimed in claim 14 or claim 15 wherein at least one of the immobilised enzymes is lipoamide dehydrogenase and the polymeric material is of polymerised polyethylene glycol acrylamide.

17. The use of the diagnostic reagent material as defined in any one of claims 1 to 16 in a method for the detection or quantitation of an analyte.

18. A method for the detection and/or quantitation of an analyte which comprises the

step of bringing a sample to be tested into contact with diagnostic reagent material as defined in any one of claims 1 to 16, in the presence of one or more reagents required for the reaction.

19. A method as claimed in claim 18 wherein the reagents are selected from indicators, e.g. chromogenic or fluorogenic dyes, substrates, redox substrates, e.g. NADH or NADPH, buffers, stabilisers, e.g. EDTA, and additional catalysts, e.g. redox catalysts.

20. A method as claimed in claim 18 or claim 19 wherein the sample to tested is a biological sample.

21. A method as claimed in claim 20 wherein the biological sample is a sample of urine, whole blood, sweat or saliva.

22. A method as claimed in any one of claims 1 to 21 which is used to detect/quantitate levels of one or more transaminases or glucose.

23. A kit comprising diagnostic reagent material as defined in any one of claims 1 to 16, together with one or more reagents suitable for carrying out one or more diagnostic, detection or quantitation assays.